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Effects of a Chinese Herbal Health Formula, "Gan-Lu-Yin", on Angiogenesis

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According to the known effects of each ingredient, Gan-Lu-Yin (GLY), a traditional Chinese herbal formula, has the potential to be an antiangiogenic agent. The purpose of this study was to explore the putative effect of GLY on antiangiogenesis. An ethanol extract of GLY was tested on chicken chorioallantoic membrane (CAM) and human umbilical vein endothelial cells (HUVEC) to evaluate the effects of GLY extract on cell proliferation, migration, and tube formation. The results showed that treatment with 1.0 mg/mL of GLY extract could markedly reduce cell migration and in vitro tube formation of HUVEC, and 1.5 mg/mL of GLY extract was sufficient to inhibit proliferation of HUVEC. The expression level of vascular endothelial growth factor (VEGF) of HUVEC was significantly decreased by 1.5 and 2.0 mg/mL of GLY extract. In chicken CAM assay, all tested concentrations of GLY extract were found to reduce the capillary mesh on the CAM of fertilized eggs. The inhibitory effects of GLY extract (1 mg/mL) were also found on tumor cell-induced HUVEC proliferation and tube formation. These observations suggested that GLY extract has an inhibitory effect on angiogenesis, which in turn may prevent tumor growth, and its mechanism might be partially associated with blocking VEGF protein expression of HUVEC.

KEYWORDS: Angiogenesis; Gan-Lu-Yin; vascular endothelial cells

INTRODUCTION

The traditional chemotherapeutic agents are usually aimed at interfering with the cellular replication of tumors cells, and therefore these drugs also damage the normally dividing cells of rapidly regenerating tissues. Furthermore, multiple drug resistances of tumor cells are still a major cause of anticancer therapy failure. Conversely, vascular endothelial cells are suitable targets for antitumor treatment because they are genetically more stable and less resistant to anticancer therapies than tumor cells (*1*, *2*). Angiogenesis, a physiological process generating new blood vessels from pre-existing ones, is well-known from embryonic development, wound healing, tissue regeneration, and tumor progression (*3*−*5*). Pathophysiological tumor neovascularization is a critical process especially on tumors that grow larger than 1−2 mm in diameter, which will largely promote tumor mass and metastasis (*3*). Thereby, tumor angiogenesis has been considered as an especially useful target to emerge as a possible therapeutic strategy with comprehensive effects to fight all solid cancers. Currently, more than 80 antiangiogenic agents are under clinical investigation for cancer treatment in phase I or phase II clinical trials (*6*, *7*). Among these, one type comprises small molecule inhibitors or monoclonal antibodies that inhibit several tyrosine kinase receptors such as vascular endothelial growth factor (VEGF) receptor; the other type comprises endogenous angiogenesis inhibitors such as Angiostatin and endostatin. Angiogenesis inhibitors have been shown to prolong progression-free survival but to have only a small effect on overall survival in patients with cancer. Thereby, a more potent antiangiogenic agent should be required to achieve the acceptable clinical and oncologic outcome.

Gan-Lu-Yin (GLY), a Chinese herbal formula, consists of *Rehmannia glutinosa*, *Liriope spicata* (Thunb.) Lour, *Eriobotrya japonica* (Thunb.) Lindl., *Citrus sinensis* Osbeck, *Glycyrrhiza uralensis* Fisch, *Artemisia capillaris* Thunb., *Dendrobium nobile* Lindl., and *Scutellaria baicalensis* Georgi. Many of the GLY components, such as *R. glutinosa*, *L. spicata* (Thunb.) Lour, *E. japonica* (Thunb.) Lindl., *C. sinensis* Osbeck, *G. uralensis* Fisch, *A. capillaris* Thunb., *D. nobile* Lindl., and *S. baicalensis* Georgi, have been used as health foods for a long history in ancient China and even nowadays. In addition, GLY has been used as a popular drink to cope with constipation or oral ulcers in older days. This formula is used to expel the heat, remove the dampness, resolve inflammation, and clean the blood according to traditional Chinese medicinal prescriptions, Tai Ping Hui Min He Ji Ju Fang. Although numerous studies mentioned that some of the single ingredients within the GLY formula have biological effects on antitumor potential by inhibiting cell proliferation or inflammation (*8*−*10*), the effect of GLY formula on

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Table 1. HPLC Separation Conditions for Identifying Marked Components within GLY[a]

compound	mobile phase	wavelength (nm)	RT (min)	contents (mg/g of GLY)
baicalein	ACN/H_3PO_4 (0.11%, pH 2.2) = 28:72	276	6.21	439
chlorogenic acid	ACN/H_3PO_4 (0.11%, pH 2.2) = 15:85	330	5.53	3
glycyrrhizic acid	ACN/H_3PO_4 (0.11%, pH 2.2) = 35:65	250	12.66	10

[a] ACN, acetonitrile; RT, retention time. All samples were loaded at a total volume of 10 μL into the HPLC cartridge; a flow rate of 1.0 mL/min was used to perform HPLC analysis.

antiangiogenesis was still unclear. Thereby, the present study was designed to explore the antiangiogenic activities of GLY on vascular endothelial cells and to evaluate the inhibitory effect of GLY on tumor cell-induced angiogenic processes.

MATERIALS AND METHODS

Materials. The ingredients of GLY, *R. glutinosa* (rehmannia), *L. spicata* (Thunb.) Lour (lilyturf), *E. japonica* (Thunb.) Lindl. (loquat leaves), *C. sinensis* Osbeck (sweet orange), *G. uralensis* Fisch (licorice root), *A. capillaris* Thunb. (capillaris), *D. nobile* Lindl. (dendrobium), and *S. baicalensis* Georgi (baical skullcap root), were provided from the Pharmacy Department of China Medical University Hospital, Taichung, Taiwan. Matrigel Basement Membrane Matrix was purchased from BD Biosciences (Bedford, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of GLY Extract. The ingredients of the GLY formula were equally weighed (about 1 kg) and soaked in 10 L of 50% ethanol solution (extractive solvent) for 3 days at room temperature. The solid residue of the above soaked herbs was filtered and discarded through a Buchner funnel lined with Whatman filter paper, and the filtrate was concentrated to paste by distillation under reduced pressure. The series concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract were further diluted with deionized water for the subsequent studies.

HPLC Analysis and Mass Spectrometry of GLY Extract. Before analysis by HPLC, GLY extract was filtered through a 0.2 μm Millipore filter, and then a total volume of 10 μL was loaded into the HPLC column. In addition, external standards were prepared at a concentration of 100 μg/mL in HPLC grade methanol and used to calculate the concentration of examined compounds. Reverse phase HPLC was performed on a Hitachi HPLC system (Tokyo, Japan) equipped with a Hitachi L-7100 pump, a Hitachi L-7400 UV detector, and a Hitachi L-7200 autosampler. Separations were accomplished on a LiChroCART 250-4 C18 HPLC cartridge (5 μm; Merck, Whitehouse Station, NJ). The separation conditions of HPLC analysis for examined compounds are described in **Table 1**. According to the HPLC separation conditions of standard compounds, three candidate fractions (collection period, retention time ± 5 min) were harvested by the HPLC system. These collected fractions were further examined by an electrospray ionization−ion trap mass spectrometry (ESI-ion trap MS) system (HCT ultra PTM Discovery system; Bruker Daltonics, Billerica, MA) to identify the marked (or potential) compounds within GLY extract. Capillary voltage was 4000 V, capillary exit offset was 220 V, skimmer potential was 40 V, and the trap drive value was 78. Conventional ESI-MS data were recorded using a scan range of m/z 200−1000. Nebulizer (nitrogen) pressure was 10 psi, dry gas (nitrogen) flow was 5 L/min, and dry temperature was 300 °C.

Cell Culture. Human umbilical vein endothelial cells (HUVEC; BCRC no. H-UV001) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured on gelatin-coated 100 mm dishes and maintained in M199 medium (31100-035; Invitrogen, Carlsbad, CA) containing 10% FBS (10099-141; Invitrogen), 30 μg/mL endothelial cell growth supplements (E0760; Sigma-Aldrich), 25 units/mL heparin, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfates. Cells at passage 3−5 were used for experiments. CAL-27 cells were obtained from American Type Culture Collection (CRL-2095; Manassas, VA) and cultured in GIBCO Dulbecco's modified Eagle's medium (12800-017; Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT), 4 mM L-glutamine, 3.7 g/L sodium bicarbonate, 100 units/mL penicillin G, and 100 μg/mL streptomycin. All cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C, and culture medium was changed every 2 days.

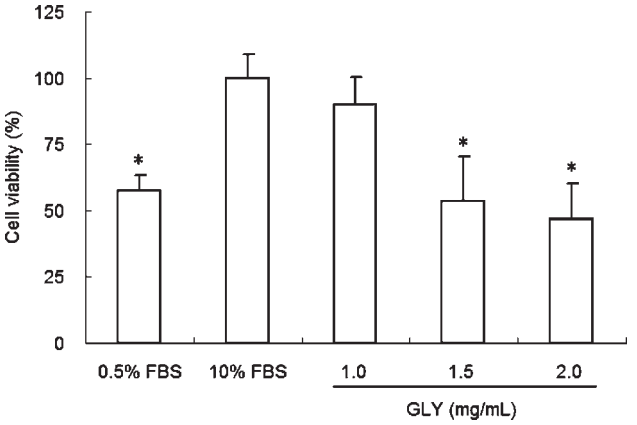

Figure 1. Inhibitory effect of Gan-Lu-Yin (GLY) extract on cell proliferation of human umbilical vein endothelial cells (HUVEC). HUVEC were incubated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY for 24 h of treatment, and cell viability was measured by MTT cell proliferation assay. The percentage of cell viability was calculated according to the values of control group (10% FBS treated group) as 100%. Histograms of all values are expressed as the mean ± SD; * indicates $p < 0.05$ as compared with the control group.

MTT Cell Viability Assay. The MTT assay was performed to measure the cytotoxicity of GLY on HUVEC. Cells were seeded in 96-well plates with 1×10^4 cells/well in culture medium overnight. After 24 h of starvation, cells were exposed to different concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract in a total volume of 100 μL of 10% FBS contained culture medium for 24 h. After 24 h of treatment, 10 μL of 5 mg/mL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added into each well. After 3 h of incubation, the cells were washed twice with iced 1× PBS, and 100 μL of DMSO was added to each well. Absorbance values at 570 nm were determined for each well using 650 nm as the reference wavelength. The absorbance can be correlated to the percentage of vital cells by comparing the data of the doped cells with those of the control group. To analyze the inhibitory effect of GLY extract on tumor cell-induced HUVEC viability, the serum-free condition medium, which was cultured with Cal-27 cells for 96 h, was harvested and centrifuged. HUVEC were cultured with harvested condition medium of Cal-27 cells and treated with different concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract for 24 h. The inhibitory effect of GLY extract on tumor cell-stimulated cell viability was measured by MTT assay.

Cell Cycle Analysis. Treated HUVEC were harvested and washed with cold 1× PBS and then fixed with 70% ice-cold ethanol overnight. After ethanol was removed by centrifuge, pellets were resuspended in 500 μL of DNA staining buffer containing 4 μg/mL of propidium iodide, 1% (v/v) Triton X-100, and 0.1 mg/mL of RNase A and incubated for 30 min at room temperature in the dark, followed by flow cytometry using FACSCanto (BD Biosciences, San Jose, CA). The cell cycle profile was analyzed using the ModFit LT Program (Verify Software House, Topsham, ME).

Wound-Healing Analysis. Cells (2.5×10^4 cells/well) were seeded on a 12-well plate in growth medium overnight. After 24 h of cell starvation, a pipet tip was used to scratch the cell monolayer to create the wound. The cells were cultured in 0.5% FBS-containing medium and treated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract. The cell number within the scratch region was recorded at 0, 6, 12, and 24 h after treatment. The migration inhibition was presented as cell density, which was calculated according to the average number of cells per 1 mm square at 0 h of each group.

In Vitro Tube Formation Assay. The wells of a 96-well plate were precoated with matrigel. After polymerization of the matrix at 37 °C,

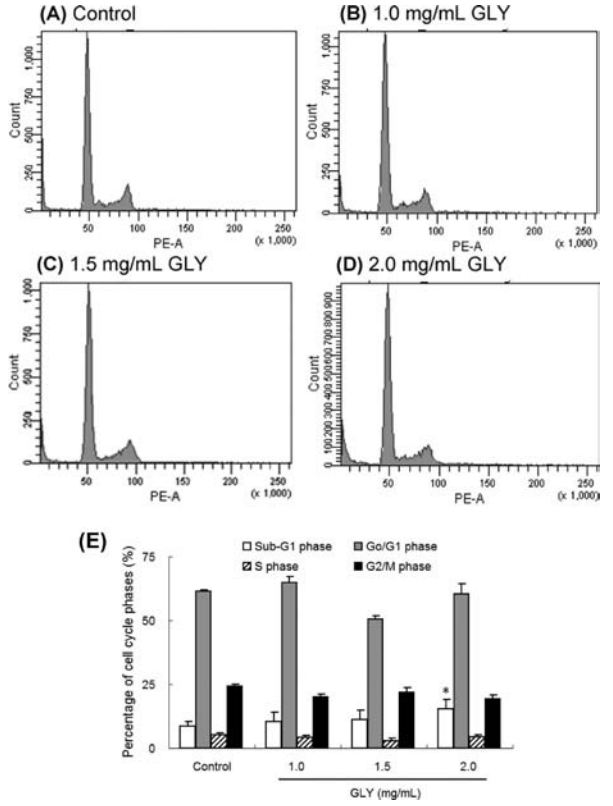

Figure 2. Effect of GLY extract on cell cycle distribution of HUVEC. HUVEC were incubated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY for 24 h of treatment, and cell cycle distribution was analyzed by flow cytometry (**A**−**D**). The percentage of cell cycle phase of each treatment is presented as a histogram (**E**), and all values are expressed as the mean ± SD; ∗ indicates $p < 0.05$ as compared with the control group.

HUVEC were then plated at a density of 1×10^4 cells/well and further incubated alone or in the presence of various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract. After 16 h of treatment, the capillary-like tube formation was observed under an inverted, phase-contrast photomicroscope, and the vessel length of the capillary mesh was measured to evaluate the antiangiogenic effect of GLY extract in tube formation. To analyze the inhibitory effect of GLY extract on tumor cell-induced tube formation of HUVEC, the serum-free condition medium, which was cultured with Cal-27 cells for 96 h, was harvested and centrifuged. HUVEC were cultured with harvested condition medium of Cal-27 cells and treated with different concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract for 24 h. The inhibitory effect of GLY extract on tumor cell-stimulated angiogenesis was measured.

Chorioallantoic Membrane (CAM) Assay. The contribution of GLY extract to angiogenesis was investigated ex vivo using the chick embryo CAM assay. Briefly, fertilized chicken eggs (10 eggs/group) were incubated at 37 °C and 80% humidity. On the eighth day of incubation, a square window was opened in the shell, and CAMs were injected with different concentrations (1.0, 1.5, and 2.0 mg/g egg) of GLY extract by insulin syringe. The window was sealed with a transpore tape after injection, and eggs were returned to the incubator. After 48 h of incubation, CAM arterious branches in each treatment group were photographed and counted by using a Nikon digital camera system (Chiyoda-ku, Tokyo, Japan). The antiangiogenic effect of GLY was presented as relative number of arterious branches.

Immunoblotting of VEGF Protein. HUVEC were cultured in 10% FBS-containing medium and treated with 1.5 or 2.0 mg/mL of GLY extract for 24 h. HUVEC were harvested for examination of the expression of cytosolic VEGF protein. The treated HUVEC were washed with 1× PBS and lysed by adding the appropriate volume of PRO-PREP protein extraction solution (17081; iNtRON Biotechnology, Gyeonggi-Do, South Korea). The cell lysate was then centrifuged at 13000*g* at 4 °C for 10 min, and the supernatant was collected for Western blot analysis. Protein concentration was measured by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Aliquots containing 50 μg of protein were separated by 10% slab SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA), and Ponceau S was used to identify the successful transfer of proteins to the membrane. Briefly, nonspecific binding sites were blocked by incubating membranes in 5% nonfat milk. Primary antibodies against VEGF (sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (ab6276; Abcam, Cambridge, MA) were diluted 1:500 and 1:5000, respectively. The secondary antibody (ab6728; Abcam) was applied using a dilution of 1:2000. Substrates were visualized using SuperSignal West Pico Chemiluminescent Substrate (34078; Thermo Scientific, Newington, NH), and the luminescence signal was acquired and analysis by a Fujifilm LAS-4000 system (San Leandro, CA).

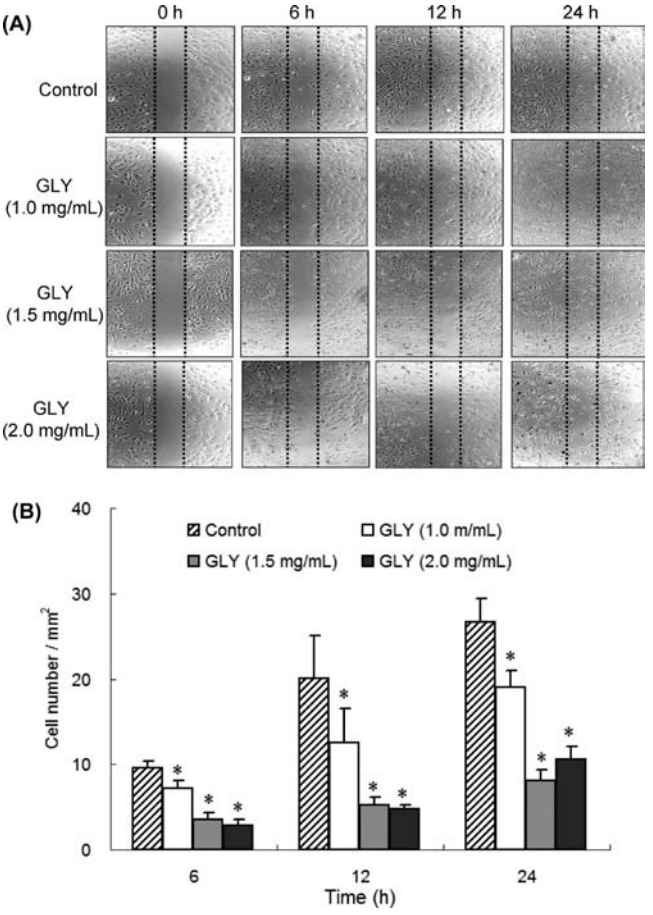

Figure 3. Inhibitory effect of GLY extract on cell migration of HUVEC. HUVEC were cultured in 0.5% FBS-supplemented medium and incubated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY, and cell migration was examined at 0, 6, 12, and 24 h after treatment by wound healing (**A**). The migration inhibition is presented as cell density, calculated according to the average number of cells per 1 mm square at 0 h of each group (**B**). ∗ indicates $p < 0.05$ as compared with the control group (0.5% FBS treated group) at the same time point.

Statistical Analysis. All data are presented as mean ± SD. In vitro experiments were done in triplicate. Statistical significance was evaluated by one-way ANOVA. A value of $p < 0.05$ was regarded as being statistically significant.

RESULTS

Inhibitory Effect of GLY Extract on Cell Proliferation of HUVEC. The antiproliferative effect of GLY was analyzed on

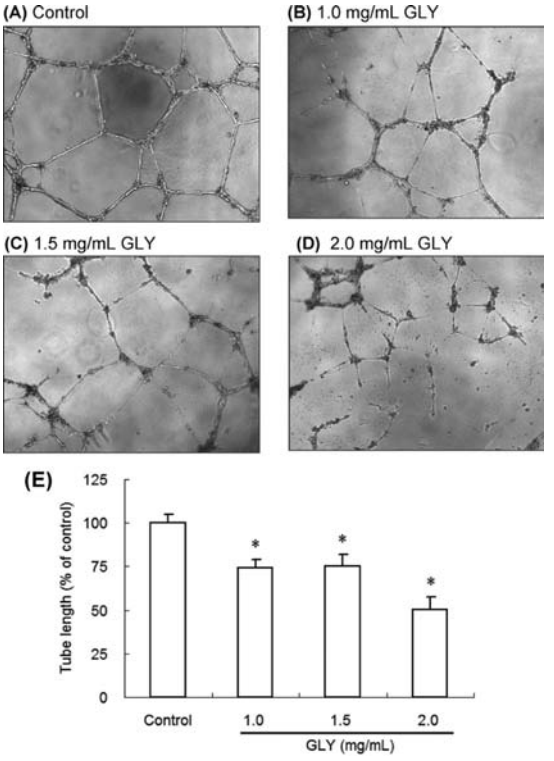

Figure 4. Inhibitory effect of GLY extract on tube formation of HUVEC. HUVEC were cultured on matrigel matrix and incubated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY, and capillary-like tube formation was examined at 16 h after treatment (**A**−**D**). Tube formation is presented as relative tube length, calculated according to the average tube length between branch nodes (**E**) and the values of the control group (10% FBS treated group) as 100%. ∗ indicates $p < 0.05$ as compared with the control group.

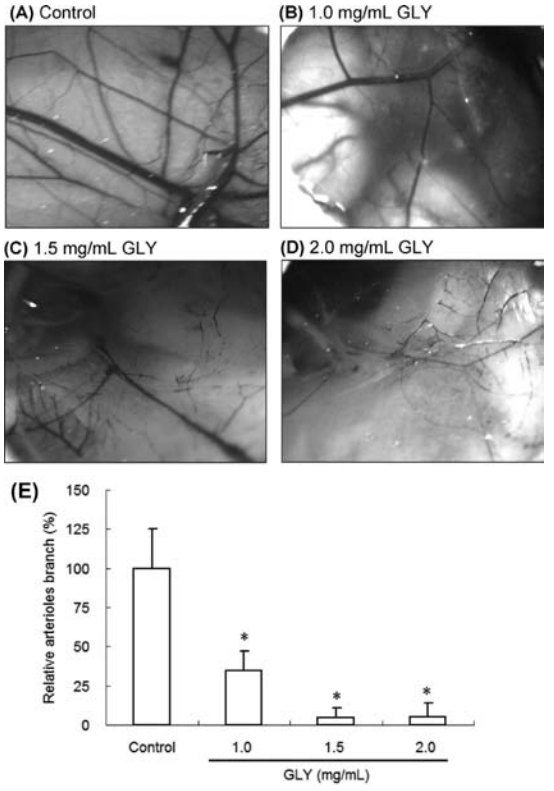

Figure 5. Ex vivo antiangiogenic effect of GLY extract was examined by chick chorioallantoic membrane (CAM) assay. The various concentrations (1.0, 1.5, and 2.0 mg/g by egg weight) of GLY were injected into the CAM of fertilized chicken eggs on day 9 of development, and the antiangiogenic effect of GLY was observed at 48 h after injection (**A**−**D**). The antiangiogenic effect of GLY is presented as relative number of arterious branch (**E**). ∗ indicates $p < 0.05$ as compared with the control group (PBS-treated group).

HUVEC treated with 10% FBS to stimulate cell growth. After 24 h of treatment with GLY extract, the results showed that 10% FBS stimulation could largely increase cell proliferation of HUVEC, and this growth effect of cells could be markedly attenuated by treatment of GLY extract (**Figure 1**). In the present study, GLY extract was found to inhibit HUVEC proliferation in a dose-dependent manner, and the effective concentration of GLY extract was measured at a dosage more than 1.5 mg/mL, which could significantly decrease HUVEC proliferation as compared with the control group ($p < 0.05$; $n = 3$).

Effect of GLY Extract on Cell Cycle Distribution of HUVEC. The cell cycle distribution of HUVEC was examined by flow cytometry on cells treated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract for 24 h (**Figure 2**). Our result revealed that GLY extract did not influence cell cycle phases including G_0/G_1, S, and G_2/M phases of HUVEC as compared to those of the control group (PBS-treated only). However, we found that the proportion of sub-G1 phase (apoptosis) of HUVEC was significantly increased for cells incubated with 2.0 mg/mL of GLY extract for 24 h ($p < 0.05$; $n = 3$).

Inhibitory Effect of GLY Extract on Cell Migration of HUVEC. The wound-healing assay was performed to evaluate dose- and time-dependent effects of GLY extract on cell migration of HUVEC (**Figure 3A**). The data showed that all of the examined concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract could markedly decrease cell migration of HUVEC at all observation times (6, 12, and 24 h) as compared with the control group (**Figure 3B**) ($p < 0.05$; $n = 3$), indicating that the GLY extract could inhibit cell migration of HUVEC in a dose- and time-dependent manner.

Inhibitory Effect of GLY Extract on Tube Formation of HUVEC. HUVEC were cultured on matrigel matrix to promote tube-like network formation, and serial concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract were used to treat HUVEC at the same time to examine the inhibitory effect on tube formation of HUVEC (**Figure 4**). Our result showed that a capillary-like tube network of HUVEC was evidently formed in the control group, and the GLY extract markedly inhibited tube formation of HUVEC in a dose-dependent manner (**Figure 4E**) as compared with the control group ($p < 0.05$; $n = 3$).

Ex Vivo Antiangiogenic Effect of GLY Extract Examined by Chick CAM Assay. The fertilized chick eggs at day 9 of embryo development were applied to test the antiangiogenic effect of GLY extract (**Figure 5**). The result revealed that angiogenesis of fertilized eggs was clearly observed after 48 h of treatment, and various concentrations (1.0, 1.5, and 2.0 mg/g by egg weight) of GLY extract could largely reduce the angiogenesis on the chorioallantoic membrane of fertilized eggs (**Figure 5E**) as compared to those of the control group ($p < 0.05$; $n = 10$).

Inhibitory Effect of GLY Extract on VEGF Expression of HUVEC. HUVEC were treated with 1.0, 1.5, or 2.0 mg/mL of GLY extract for 24 h, and HUVEC were harvested to examine the cytosolic expression of VEGF protein (**Figure 6**). The result indicated that both 1.5 and 2.0 mg/mL of GLY extract could markedly decrease cytosolic VEGF protein of HUVEC as compared with the control group.

Inhibitory Effect of GLY Extract on Tumor Cell-Induced HUVEC Proliferation. The cell growth of HUVEC was stimulated with harvested condition medium of Cal-27 tumor cells

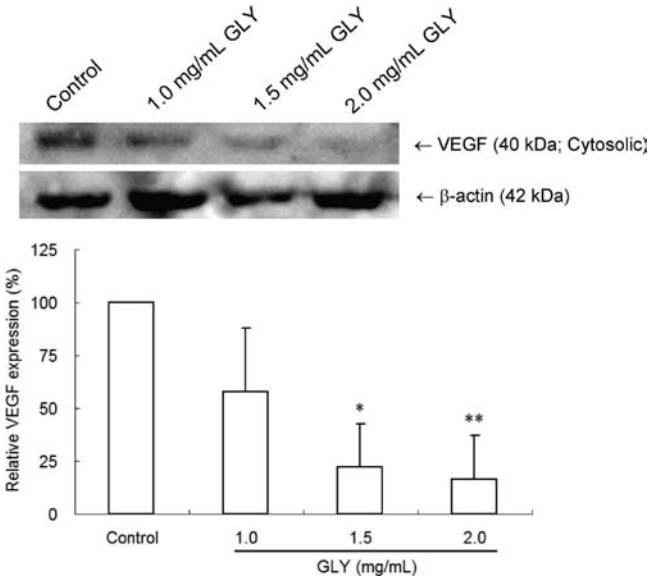

Figure 6. Inhibitory effect of GLY extract on cytosolic VEGF expression of HUVEC. HUVEC were cultured in 10% FBS-supplemented medium and incubated with 1.0, 1.5, and 2.0 mg/mL of GLY, and HUVEC were harvested to examine the intracellular VEGF protein of HUVEC after 24 h of treatment. The results for cytosolic VEGF were normalized to the band density of internal control (β-actin), and the relative protein expression of cytosolic VEGF was calculated according to the values of the control group (cultured medium treated only) as 100%. $*$ and $**$ indicate $p < 0.05$ and $p < 0.01$ as compared with the control group, respectively.

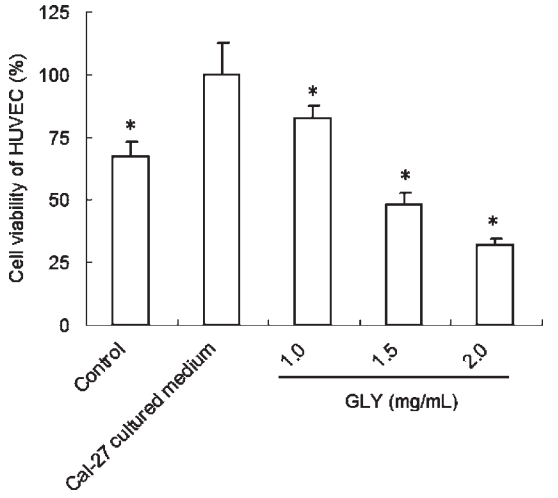

Figure 7. Inhibitory effect of GLY extract on tumor cell-induced HUVEC proliferation. The serum-free condition medium of Cal-27 cancer cell was harvested, and HUVEC were stimulated with collected cultured medium and then cotreated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract. The inhibitory effect of GLY was examined at 24 h after treatment of GLY extract by MTT assay. The percentage of cell viability was calculated according to the values of the control group (cultured medium treated only) as 100%. Histograms of all values are expressed as the mean $\pm$ SD; $*$ indicates $p < 0.05$ as compared with the control group.

while cells were treated with different concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract (**Figure 7**). In the present study, the condition medium of Cal-27 cells could significantly expand the cell number of HUVEC after 24 h of stimulation, which could be markedly reversed by treatment of GLY extract in a dose-dependent manner as compared with the control group ($p < 0.05$; $n = 3$).

Inhibitory Effect of GLY Extract on Tumor Cell-Induced Tube Formation of HUVEC. The tube-like network formation of HUVEC was induced by condition medium of Cal-27 tumor cells while cells were incubated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract (**Figure 8**). The experimental result showed that capillary-like tube formation of HUVEC was evidently observed after 24 of treatment, and these tube-like networks could be markedly attenuated as cells incubated with GLY extract at the same time (**Figure 8E**) ($p < 0.05$; $n = 3$).

Qualification of Extraction Procedure of GLY by HPLC Analysis and Mass Spectrometry. Some of the marked compounds (e.g., baicalein, chlorogenic acid, and glycyrrhizic acid) from a single ingredient within the GLY formula were identified by HPLC to be indicator compounds for quality check of extraction procedure of each batch (**Figure 9A**). Using HPLC quantification, the contents of baicalein, chlorogenic acid, and glycyrrhizic acid were calculated to be 439, 3, and 10 mg/g of GLY extract, respectively (**Table 1**). In addition, on the basis of the retention time of standard compounds, we collected three HPLC fractions (collection period, retention time $\pm$ 5 min) that might contain baicalein, chlorogenic acid, and glycyrrhizic acid, respectively. These harvested fractions were further examined by ESI-MS to identify the marked compounds (baicalein, chlorogenic acid, and glycyrrhizic acid) of GLY extract. The results of ESI-MS evidenced that there were signals at mass-to-charge ratios (m/z) of 270.9, 355.0, and 823.37, which were consistent with the expected masses of baicalein ($M_w \approx 270.24$), chlorogenic acid ($M_w \approx 354.31$), and glycyrrhizic acid ($M_w \approx 822.93$), respectively (**Figure 9B**). In the present experiment, the data from HPLC/ESI-MS indicated that the three marked components,

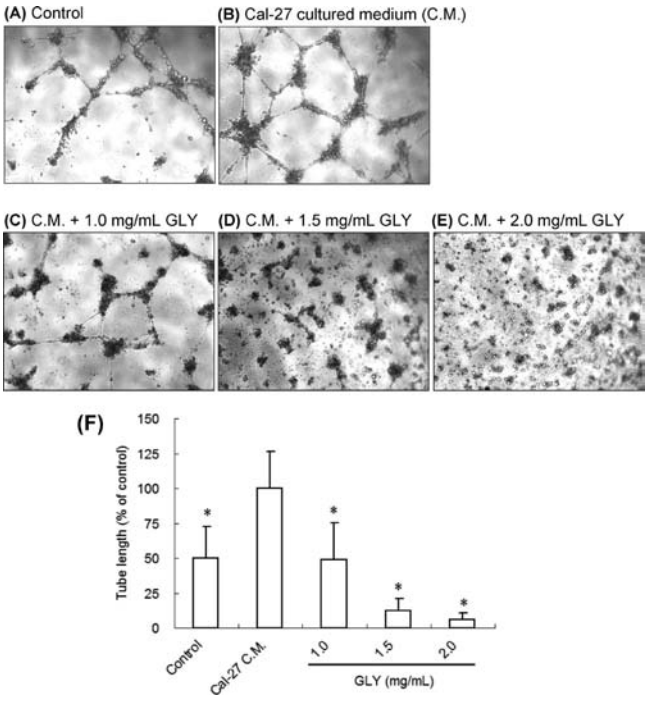

Figure 8. Inhibitory effect of GLY extract on tumor cell-induced tube formation of HUVEC. HUVEC were cultured on matrigel matrix, stimulated with collected cultured medium (C.M.) of Cal-27 cells, and then cotreated with various concentrations (1.0, 1.5, and 2.0 mg/mL) of GLY extract for 24 h (**A**−**D**). The inhibition of GLY on tumor cell-induced tube formation was examined and is presented as relative tube length, calculated according to the average tube length between branch nodes (**E**) and the values of the control group (cultured medium treated only) as 100%. $*$ indicates $p < 0.05$ as compared with the control group.

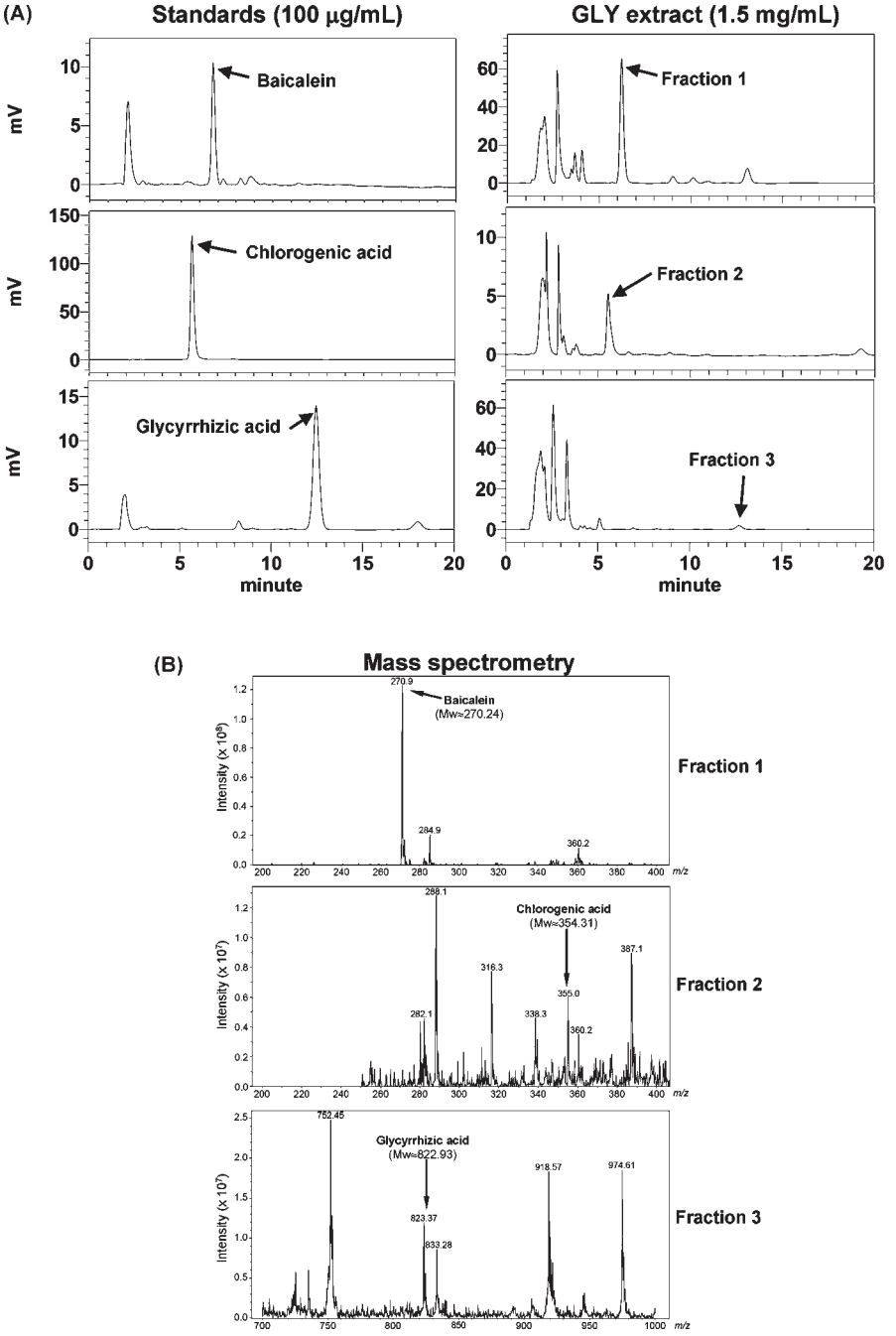

Figure 9. Representative HPLC and mass spectrometry of the marked compounds in GLY extract. The right and left chromatograms indicate HPLC separation of the standard compounds (100 μg/mL) and GLY extract (1.5 mg/mL), respectively (**A**). A total volume of 10 μL was loaded into the HPLC column to measure the relative content of baicalein, chlorogenic acid, and glycyrrhizic acid in GLY extract according to the concentration of standard compounds. The examination conditions and monitoring wavelength of HPLC analysis were described in **Table 1**. Three HPLC fractions (collection period, retention time $\pm$ 5 min) were collected according to the separation conditions of standard compounds, and these fractions were further identified by ESI-MS to confirm the marked compounds (baicalein, chlorogenic acid, and glycyrrhizic acid) within the GLY extract (**B**).

baicalein, chlorogenic acid, and glycyrrhizic acid, of GLY extract must be contained in the harvested HPLC fractions, respectively.

DISCUSSION

Angiogenesis is a very complicated process that includes at least four sequential steps: endothelial cell proliferation, degradation of basement membrane by proteinase, migration of endothelial cells, and tube and new basement membrane formation, promoting blood circulation. We examined the effect of GLY extract on antiangiogenesis by using in vitro and ex vivo angiogenesis assays. GLY extract could inhibit cell proliferation of HUVEC stimulated by serum or tumor cells (**Figures 1** and **7**). The cell migration of HUVEC was also attenuated by GLY treatment (**Figure 3**). Similarly, GLY extract could also reduce in vitro tube formation of HUVEC stimulated with matrigel only or combined with cultured medium from Cal-27 tumor cells (**Figures 4** and **8**). In an ex vivo study, we found that GLY has an effect on antiangiogenesis on chorioallantoic membrane of fertilized eggs (**Figure 5**). On the basis of these results, treatment of GLY has been evidenced that it could interfere with the angiogenic process at the step of cell proliferation, migration, and tube formation of vascular endothelial cells.

Recently, the work by Lin et al. mentioned that at least 14 main components of GLY have been identified using liquid chromatography−mass spectrometry (LC-MS) and inductively coupled plasma MS (ICP-MS), and these components included baicalin, baicalein, oroxylin A-7-*O*-glucuronide, wogonin-7-*O*-glucuronide, wogonin, and oroxylin A in *Radix Scutellariae*; naringin and neohesperidin in *Aurantii fructus*; and liquiritigenin, liquiritin, and glycyrrhizic acid in *Radix Glycyrrhizae* (*11*). In our study, the indicator components of GLY extract such as baicalein, chlorogenic acid, and glycyrrhizic acid have been detected by HPLC/ESI-MS analysis (**Figure 9**). It has been mentioned that baicalin and baicalein, two flavonoid compounds, have inhibitory effects on endothelial cell proliferation, migration, and differentiation (*12*, *13*). Endothelial cell proliferation, adhesion, and migration are early essential events for mediating angiogenesis. Hsieh et al. indicated that baicalein significantly up-regulated protein expression of integrins and vinculin in rat endothelial cells, which will result in the increase of focal contact formation and cell adhesion to fibronectin and vitronectin. Coordination and balance between cell adhesion to and detachment from extracellular matrix (ECM) is crucial for cell motility, so unilateral increasing cell adhesion will severely affect endothelial migration and angiogenesis processes. In addition, it has been reported that baicalein and baicalin could inhibit cell proliferation of tumor cells and induce apoptosis of myeloma cells (*14*, *15*). In the present study, we found GLY extract could induce apoptosis of vascular endothelial cells in a dose-dependent manner (**Figure 2**). Sun et al. found that oroxylin A suppresses invasion through inhibiting cell migration and down-regulating the expression of matrix metalloproteinase-2/9 in human breast cancer cells (*16*). Wogonin, a natural monoflavonoid, has been evidenced to inhibit inflammatory cytokine-induced angiogenesis of HUVEC and suppress the VEGF-stimulated migration and tube formation of HUVEC (*17*, *18*). Schindler et al. reported that naringin could decrease tumor-induced vascular proliferation by reducing the release of VEGF from human tumor cells (*19*). The inhibitory effect of liquiritigenin on tube formation of vascular endothelial cells has also been reported (*20*). However, glycyrrhizic acid, the main component of licorice, has been investigated for its ability to increase tube formation (*20*). Relf et al. mentioned that caffeic acid and its derivative, CAPDE, can suppress tumor angiogenesis by blocking VEGF expression in human renal carcinoma cells (*21*). Although chlorogenic acid is also a caffeic acid derivative compound, so far there has not been direct evidence to point out the association with the effect on antiangiogenesis. However, previous literature indicated that it is a new type of strong matrix metalloproteinase-9 inhibitor, which might prevent the invasion and metastasis of malignant cancer cells (*22*).

Endothelial cells are the major target for VEGF, which is a critical regulatory factor that modulates multiple endothelial cell functions, including cell migration, proliferation, and angiogenesis (*23*, *24*). Thereby, recently research directions and therapeutic strategies of clinical chemotherapy have focused on inhibiting or neutralizing VEGF or its receptors, blocking endothelial cell activation, reducing matrix metalloproteinases, and suppressing tumor vasculature (*6*, *25*−*27*). In the present study, we found GLY extract could attenuate VEGF expression of endothelial cells (**Figure 6**), and this effect might partially explain why GLY extract could significantly affect several biological functions of endothelial cells on cell survival, migration, and tube formation.

Tumor-induced pathological angiogenesis was strongly associated with release of pro-inflammatory factors and cytokines, which have been reported to be partially secreted by cancer cells to promote tumor neovascularization (*21*, *28*−*30*), and these angiogenic factors or their receptors have been targets of angiogenesis inhibitors (i.e., Avastin and Tarceva) applied in molecular target therapy of clinical cancer treatment. In our study, we found that cultured medium of Cal-27 cells could evidently increase cell proliferation and tube formation of HUVEC, and this result suggested that some proliferative or angiogenic factors could be released into condition medium under long-term cultivation of tumor cells. Several studies have reported that baicalein and naringin could reduce VEGF expression in cancer cells (*19*, *31*), which might partially explain why GLY extract could markedly attenuate tumor cell-induced cell proliferation and tube formation of HUVEC (**Figures 7** and **8**).

In summary, our experimental results revealed that ethanol extract of GLY could markedly inhibit cell viability, migration, in vitro tube formation, and VEGF expression of HUVEC. Besides, GLY could also attenuate the proliferation and capillary-like mesh of HUVEC stimulated by cultured medium from tumor cells. In the result of ex vivo examination, angiogenesis of the chorioallantoic membrane of fertilized eggs was also suppressed by GLY treatment. According to the studies of well-known compounds, GLY extract might use four approaches to perform its antiangiogenesis effect. First, increasing endothelial cell adhesion via up-regulation of integrins and vinculin inhibits endothelial migration, as performed by baicalein. Second, the effects of angiogenic factors on cell proliferation, migration, and tube formation of endothelial cells can be blocked by liquiritigenin, and wogonin. Third, baicalein and naringin may reduce VEGF expression of tumor cells. Fourth, suppression of matrix metalloproteinase activity to prevent cell migration can be achieved by chlorogenic acid. On the basis of these results, we propose that the ethanol extract of GLY has the potential to be an effective pharmacological reagent in preventing cancer-related angiogenesis.

ABBREVIATIONS USED

GLY, Gan-Lu-Yin; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

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